PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 39/395, 37/02, 37/04
A61K 35/54, C12P 21/06
C07K 15/06, 15/14, C12N 15/10
C12N 15/12

A1

(11) International Publication Number: WO 93/14786

(43) International Publication Date: 5 August 1993 (05.08.93)

(21) International Application Number: PCT/US93/01038

(22) International Filing Date: 4 February 1993 (04.02.93)

(30) Priority data:
830,627 4 February 1992 (04.02.92) US

(71) Applicant: COLORADO STATE UNIVERSITY RESEARCH FOUNDATION [US/US]; 601 South Howes, Fort Collins, CO 80521 (US).

(72) Inventor: BOWEN, Richard, Arnold ; 5036 East Country Road 60, Wellington, CO 80549 (US).

(74) Agents: KOVARIK, Joseph, E. et al.; Sheridan Ross & McIntosh, 1700 Lincoln Street, 35th Floor, Denver, CO 80203 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, UA, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: COMPOSITION AND METHOD TO PREVENT CONCEPTION OR TO CAUSE STERILITY IN ANIMALS

(57) Abstract

The present invention provides zona pellucida protein-based contraceptive vaccines and methods to use zona pellucida protein vaccines to prevent conception in animals. Contraception may be either reversible (i.e., temporary) or permanent (i.e., renders an animal sterile) depending on the mode of administration of the vaccine. The present invention includes recombinant porcine zona pellucida proteins, DNA sequences encoding those proteins, recombinant methods to produce those proteins, and methods to produce vaccines comprising one or more of those proteins. The present invention also includes methods to use one or more zona pellucida proteins to induce the production of zona pellucida-specific antibodies in a vaccinated animal and to detect the presence of zona pellucida-specific antibodies in a sample of a bodily fluid.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

COMPOSITION AND METHOD TO PREVENT CONCEPTION OR TO CAUSE STERILITY IN ANIMALS

Field of the Invention

The present invention is related to protein-based contraceptive vaccines and methods for their use. More particularly, the invention relates to recombinant porcine zona pellucida proteins and their use as a contraceptive vaccine.

Background

The zona pellucida is a glycoprotein coat surrounding the eggs of mammals. Sperm normally attach to the zona pellucida via a sperm receptor located on the zona pellucida. After attachment, a sperm releases enzymes enabling it to penetrate the zona pellucida and to enter and fertilize the egg.

An immunological approach to contraception based on blocking a sperm's ability to bind to the zona pellucida is desirable because it obviates the need for mechanical contraceptive devices which are often ineffective and may cause infection. A contraceptive vaccine, which need be administered only once or a few times, is much more convenient than current modes of contraception. Use of contraceptive vaccines to reversibly or permanently sterilize animals is an attractive and economic alternative to surgical castration. A contraceptive vaccine also has advantages over hormone or hormone antagonist treatments which may cause undesirable side effects, such as cancer.

Moreover, a contraceptive vaccine is preferable to passive immunization techniques in which polyclonal or

monoclonal antibodies are administered. Techniques of passive immunization are described in U.S Patent No. 3,992,520 by Gwatkin, issued Nov. 16, 1976; U.S. Patent No. 4,996,297 by Dunbar, issued Feb. 26, 1991; Henderson et al., J. Reprod. Fert. 83:325-343, 1988. Antibodies function for only a limited time period in the body and must, therefore, be administered frequently. Repeated administration of antibodies often causes immunized individuals to mount an immune response against the antibodies themselves, rendering the treatments ineffective.

Anti-idiotypic antibodies that contain images of zona pellucida epitopes, such as those described in U.S. Patent 4,795,634 by Grimes et al., Jan. 3, 1989, are difficult and expensive to produce. Moreover, individuals immunized with such anti-idiotypic antibodies may mount immune responses against regions of the anti-idiotypic antibody other than the zona pellucida-like domain.

A source of zona pellucida proteins having the required specificity, efficacy, and absence of contaminants required for commercial applications are not known to the inventor. Purification of zona pellucida proteins has been hampered by the proteins' heterogenous glycosylation patterns and multiple disulfide bonds. Moreover, utilizing natural sources of zona pellucida proteins to produce vaccines is impractical because there are simply not enough ovaries available for commercial scale development.

Although attempts have been made to purify porcine zona pellucida proteins, it is still unclear how many distinct porcine zona pellucida proteins there are. For example, Hedrick and Wardrip (Anal. Biochem. 157:63-70, 1986) reported four porcine zona pellucida proteins with molecular weights of 90 kilodaltons (kD) (ZP1), 65 kD (ZP2), 55 kD (ZP3), and 25 kD (ZP4). However, further studies indicated that there are at least two 55-kD proteins, referred to as ZP3-alpha and ZP3-beta (Hedrick and Wardrip, Dev. Biol. 121:478-488, 1987; Yurewicz et al., J. Biol. Chem. 262:564-571, 1987) and that ZP4 and ZP2 may be proteolytic products of ZP1 (Japanese Patent JP 63,150,299 to Toa Nenryo Kogyo KK, 1988; Hedrick and Wardrip, Dev. Biol. 121:478-488, 1987).

Partially purified preparations of porcine zona pellucida proteins have been tested for their ability to induce the formation of zona pellucida-specific antibodies and to temporarily prevent conception in rabbits, dogs, mares, and several kinds of monkeys (e.g., Shivers et al., J. Am. Anim. Hosp. Assn. 17:823-828, 1981; Mahi-Brown et al., Biol. Reprod. 32:761-772, 1985; Liu et al., J. Reprod. Fert. 85:19-29, 1989; Gulyas et al., Gamete Res. 4:299-307, 1983; Sacco et al., Biol. Reprod. 36:481-490, 1987; reviewed by Henderson et al. in J. Reprod. Fert. 83:325-343, 1988). It has not been possible, however, to quantify the efficacy of such impure protein preparations to elicit short-term contraception. Early attempts to use a recombinant fusion protein produced by E. coli and

containing mouse zona pellucida protein ZP3 joined to beta galactosidase as a vaccine were unsuccessful (cited in PCT International Publication No. WO 90/15624 by Dean, published December 27, 1990).

Based on the above, it is apparent there is a need for a safe, well-characterized, efficacious, and inexpensive zona pellucida protein-based vaccine to prevent conception in animals. There is particularly a need for a zona pellucida protein-based vaccine which when administered to an animal before puberty would render the animal permanently sterile.

Summary of the Invention

The present invention provides zona pellucida protein-based contraceptive vaccines and methods to use zona pellucida protein vaccines to prevent conception in animals. Contraception may be either reversible (i.e., temporary) or permanent (i.e., rendering an animal sterile) depending on the manner in which the vaccine is administered. The present invention includes recombinant porcine zona pellucida proteins, DNA sequences encoding those proteins, recombinant methods to produce those proteins, and methods to produce vaccines comprising one or more of those proteins. The present invention also includes methods to use one or more zona pellucida proteins to induce the production of zona pellucida-specific antibodies in a vaccinated animal and to detect the presence of zona

pellucida-specific antibodies in a sample of a bodily fluid.

Brief Description of the Figures

Figure 1 illustrates the relative locations of porcine zona pellucida DNA sequences ZPDS.1711, ZPDS.535, ZPDS.411, and ZPDS.1176 on ZPDS.2500.

Figure 2 illustrates the DNA and deduced amino acid sequences of ZPDS.1711.

Figure 3 illustrates the DNA and deduced amino acid sequences of ZPDS.311.

Figure 4 illustrates the DNA and deduced amino acid sequences of ZPDS.447.

Figure 5 is a schematic drawing of recombinant molecule pGEX2T:ZPDS.411.

Figure 6 is a schematic drawing of recombinant molecule pVL1393:ZPDS.1711.

Detailed Description of the Invention

Production of recombinant porcine zona pellucida proteins

One aspect of the present invention involves the production of recombinant porcine zona pellucida proteins using nucleic acid sequences that encode at least a portion of a zona pellucida protein capable of preventing conception when administered to an animal. As used herein, "at least a portion of a zona pellucida protein " refers to a segment of a zona pellucida protein ranging in size from

at least about seven amino acids to a full-length protein that is capable of preventing conception.

The nucleic acid sequences of the present invention can be either RNA or DNA and can be used to produce recombinant zona pellucida proteins. As used herein, a "zona pellucida DNA sequence" refers to a DNA sequence corresponding to at least a portion of a zona pellucida gene that is sufficient to encode a protein capable of preventing conception when administered to an animal. Zona pellucida DNA sequences can be isolated from natural sources or can be synthesized chemically. The present invention includes zona pellucida DNA sequences that encode full-length zona pellucida proteins as well as DNA sequences that contain nucleotide deletions, additions, and/or substitutions which do not interfere with a sequence's ability to encode a protein capable of preventing conception in an animal. Preferred zona pellucida DNA sequences encode recombinant zona pellucida proteins that share significant homology with porcine zona pellucida proteins.

As used herein, a "recombinant zona pellucida protein," also called a "recombinant protein," refers to a protein obtained using the techniques of recombinant DNA technology. The minimal size of a recombinant zona pellucida protein is the shortest segment of amino acids required to induce the formation of antibodies capable of preventing conception. While not bound by theory, it is believed that a protein segment at least about seven amino

acids long is required to elicit antibody formation. A recombinant protein can be produced in a host in which at least one zona pellucida DNA sequence has been inserted in a manner such that the host is capable of expressing said sequence (i.e., of producing a protein by transcribing and translating the inserted DNA sequence). A recombinant protein can also be produced by chemically synthesizing a protein corresponding to a zona pellucida DNA sequence, or by incubating a nucleic acid sequence encoding at least a portion of a zona pellucida protein with the appropriate enzymes and substrates to express the protein.

One embodiment of the present invention involves the identification and isolation of zona pellucida DNA sequences. The zona pellucida DNA sequences of the present invention can be isolated from any species that contains DNA sequences encoding a protein corresponding to a zona pellucida protein capable of inducing contraception in an animal. Preferred species from which to isolate zona pellucida DNA sequences are mammals, and preferred mammals from which to isolate zona pellucida DNA sequences are pigs.

Methods to isolate the zona pellucida DNA sequences of the present invention include, but are not limited to, screening a complementary DNA (cDNA) or genomic DNA library with at least one oligo- or polynucleotide able to bind to zona pellucida DNA sequences; screening a cDNA expression library with a preparation of zona pellucida-specific antibodies or other compounds able to bind to the zona

pellucida; using polymerase chain reaction amplification to make multiple copies of zona pellucida DNA sequences directly from nucleic acids; and chemically synthesizing zona pellucida DNA sequences.

The preferred source of messenger RNA (mRNA) from which to construct a cDNA library is the ovary because the ovary is the only known tissue which expresses zona pellucida genes. A most preferred source of mRNA is an ovary isolated from a pig that has not yet undergone puberty, preferably a pig that is three to five months old. While not being bound by theory, it is believed that zona pellucida proteins are expressed at high levels during this phase of pig development.

Techniques for constructing cDNA and genomic libraries in either prokaryotic or eukaryotic cells are described in detail in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Labs Press, 1989). Briefly, RNA is isolated from ovaries, and polyadenylated RNA (which includes mRNA) is purified from total RNA using oligo-dT cellulose chromatography. The isolated polyadenylated RNA is used as a template to prepare cDNA in a two step process. First, reverse transcriptase and polyadenylated RNA are combined with oligo-dT, random primers, or a mixture thereof, to obtain a DNA copy of the RNA. Second, DNA polymerase and RNAse H are added to the DNA copy to produce a second DNA strand. The resulting cDNA sequences are a complex mixture of DNA sequences including those that

encode at least a portion of proteins expressed in the ovary, such as zona pellucida proteins.

The mixture of all cDNA sequences is inserted into prokaryotic or eukaryotic cloning vectors, which are subsequently transformed into prokaryotic or eukaryotic host cells, respectively. As used herein, a "vector" is a nucleic acid sequence that is capable of being transformed into a host and is capable of replicating in said host. A vector usually has one or more sites at which DNA sequences can be inserted without disrupting a vector's ability to transform or replicate. Vectors often have markers suitable for identification of hosts transformed with said vectors. Vectors include, but are not limited to plasmids, cosmids, phagemids, bacteriophage, and viruses. As used herein, "transformation" covers any process by which a nucleic acid is inserted into a cell, wherein the cell may remain unicellular or may grow into a tissue or multicellular organism. Transformation includes, but is not limited to, techniques such as transfection, electroporation, protoplast fusion, and the process by which transgenic animals are produced.

The preferred cloning vector of the present invention is an E. coli bacteriophage expression vector called lambda gt11, and the preferred host is E. coli. In a preferred embodiment, EcoRI linkers are added to the ends of porcine ovary cDNA sequences and the resultant EcoRI-linkered cDNA sequences are inserted into lambda gt11 at the EcoRI restriction endonuclease site.

An ovary cDNA library consists of numerous clones containing a variety of cDNA sequences. Only a few of the clones contain sequences corresponding to zona pellucida DNA sequences. In accordance with one embodiment of the present invention, a mammalian ovary cDNA library, preferably from a pig, is screened to identify clones containing zona pellucida DNA sequences.

cDNA or genomic DNA libraries can be screened with oligo- and polynucleotide probes that are sufficiently similar to zona pellucida DNA sequences to be able to hybridize to nucleic acids containing zona pellucida DNA sequences. The sequences of the oligo- or polynucleotides can be derived from partial zona pellucida protein amino acid sequences and may be either codon-biased or degenerate. The oligonucleotides or polynucleotides can be either RNA- or DNA-based. Zona pellucida DNA sequences produced in accordance with the present invention can be used as probes to identify additional zona pellucida DNA sequences. Methods to identify specific DNA sequences using nucleic acid probes are described in detail in Sambrook et al. (ibid.).

In another embodiment of the present invention, zona pellucida-specific antibodies or other compounds able to bind specifically to the zona pellucida are used to screen cDNA expression libraries for clones containing zona pellucida genes. As used herein, "zona pellucida-specific antibodies" refer to antibodies able to recognize and bind to zona pellucida proteins but are not able to effectively

bind to other proteins. "cDNA expression libraries" are libraries which are incubated under conditions that induce the expression of proteins encoded by DNA sequences of the cDNA library.

In a preferred embodiment, a cDNA expression library is plated onto a solidified medium such that individual colonies or plaques can be identified. The solidified medium promotes growth and production of proteins encoded by the cDNA sequences. Preferably, the production of proteins is induced after colonies or plaques have formed. The produced proteins are transferred onto a filter and exposed to zona pellucida-specific antibodies under conditions that promote binding between the antibodies and recombinant zona pellucida proteins. In order to visualize colonies or plaques producing recombinant zona pellucida proteins, zona pellucida-specific antibodies that are labeled may be used. Alternatively, a second antibody which is labeled and which binds to the zona pellucida-specific antibodies is added under conditions that promote binding of the second antibody to zona pellucida-specific antibodies already bound to recombinant zona pellucida proteins. Antibodies may be labeled in a variety of ways, including, but not limited to, the addition of radioactive, enzymatic, or fluorescent labels. Colonies or plaques producing proteins capable of binding to zona pellucida-specific antibodies may be identified by autoradiography or visual inspection and isolated. If necessary, such

colonies or plaques may be replated and rescreened until single isolates are obtained.

Antibodies which can be used to identify colonies containing zona pellucida DNA sequences include monoclonal and polyclonal antibodies and fragments thereof able to bind to zona pellucida proteins. In a preferred embodiment, a polyclonal antibody preparation obtained by immunizing an animal with solubilized mammalian, preferably porcine, zonae pellucidae is used. Zonae pellucidae may be isolated from ovaries and then solubilized by heating in an aqueous medium. Solubilized zonae pellucidae should contain all zona pellucida proteins and thus, should induce an animal to produce a variety of antibodies capable of binding to most, if not all, zona pellucida proteins. Antibodies obtained by immunizing an animal with solubilized porcine zonae pellucidae are referred to as "antibodies specific for a solubilized porcine zona pellucida." In a preferred embodiment, porcine zona pellucida DNA sequences are identified by screening a porcine ovary cDNA expression library with rabbit polyclonal antibodies specific for a solubilized porcine zona pellucida.

The present invention includes certain DNA sequences which may be used to identify additional zona pellucida-containing DNA sequences and/or to express proteins capable of sterilizing or otherwise preventing conception in an animal. Full-length or hybrid zona pellucida genes may be obtained by joining two or more isolated or synthetic zona pellucida DNA sequences together.

In accordance with the methods described above, a zona pellucida DNA sequence of about 2500 base pairs (bp) was isolated from a porcine ovary cDNA expression library which encodes a protein capable of binding to zona pellucida-specific antibodies. Referring to Figure 1, the zona pellucida DNA sequence of about 2500-bp, called ZPDS.2500, is composed of 3 EcoRI fragments of about 535 bp, about 1176 bp, and about 800 bp. ZPDS.1711 is composed of the EcoRI fragment of about 535 bp, called ZPDS.535, joined to the EcoRI fragment of about 1176 bp, called ZPDS.1176. ZPDS.411 is the HincII-EcoRI fragment of about 411 bp contained within ZPDS.535.

ZPDS.2500 encodes 305 amino acids of a porcine zona pellucida protein called rZPP.305 (see Figure 2). The coding sequence is contained within ZPDS.1711. The protein encoded by ZPDS.2500 does not share significant homology with reported DNA sequences encoding mouse ZP2 (Liang et al., Mol. Cell. Biol. 10:1507-1515, 1990), mouse ZP3 (Ringuette et al., Proc. Natl. Acad. Sci. USA 83:4341-4345, 1986; Ringuette et al., Dev. Biol. 127:287-295, 1988; Kinloch et al., Proc. Natl. Acad. Sci. USA 85:6409-6413, 1988), hamster ZP3 (Kinloch et al., Dev. Biol. 142:414-421, 1990), human ZP3 (Chamberlin and Dean, Proc. Natl. Acad. Sci. USA 87:6014-6018, 1990), or rabbit 55-kD deglycosylated zona pellucida protein (Schwoebel et al., J. Biol. Chem. 266:7214-7219, 1991; U.S. Patent No. 4,996,297 to Dunbar, 1991). Based on immunoprecipitation data, it is believed that ZPDS.2500 encodes a portion of porcine zona

pellucida protein ZP2. It has been found that administration of the recombinant zona pellucida protein encoded by ZPDS.411 to rabbits induces the production of zona pellucida-specific antibodies.

In addition to ZPDS.2500, the present invention includes two other distinct zona pellucida DNA sequences that encode proteins that bind to antibodies specific for solubilized porcine zona pellucida. These zona pellucida DNA sequences were isolated from a cDNA expression library as described above. As shown in Figure 3, ZPDS.311 is an EcoRI fragment of about 311 bp that is capable of encoding a recombinant zona pellucida protein of about 103 amino acids (rZPP.103). ZPDS.447 is an EcoRI fragment of about 447 bp capable of encoding a recombinant zona pellucida protein of about 149 amino acids, called rZPP.149 (Figure 4). Neither ZPDS.311 nor ZPDS.447 share significant homology with other zona pellucida proteins. ZPDS.311 and ZPDS.447 do not hybridize to ZPDS.2500, nor do they hybridize to each other, suggesting that the three sequences represent distinct zona pellucida DNA sequences.

Another aspect of the present invention involves the use of zona pellucida DNA sequences to produce recombinant proteins that are capable of preventing conception. At least one zona pellucida DNA sequence is transformed into a host in a manner such that the transformed host is capable of expressing a recombinant zona pellucida protein(s) encoded by said DNA sequence(s).

In another embodiment of the present invention, recombinant molecules are produced which comprise zona pellucida nucleic acid sequences, preferably zona pellucida DNA sequences, operatively linked to expression vectors. A suitable expression vector comprises a nucleic acid sequence that is capable of inserting a zona pellucida nucleic acid sequence, preferably a zona pellucida DNA sequence, into a host and of directing transcription and translation of that sequence within the host.

Expression vectors of the present invention include both prokaryotic and eukaryotic vectors including, but not limited to, those that direct gene expression in bacteria, yeast, fungi, animals, insects, and plants. Expression vectors include, but are not limited to, viruses and plasmids that contain regulatory sequences that control the expression of a gene. Examples of such regulatory sequences include, but are not limited to, the regulatory sequences of tac, lac, trp, trc bacteriophage T7, lambda, baculovirus, Heliothis zea insect virus, vaccinia virus, adenovirus, simian virus 40, retroviruses, actin, metallothionein system, yeast alpha mating factor, Pichia alcohol oxidase system, and other sequences capable of controlling gene expression in prokaryotic or eukaryotic cells.

Expression vectors of the present invention may also contain secretory signals to enable an expressed zona pellucida protein to be secreted from its host cell or may contain fusion sequences which lead to the expression of

inserted zona pellucida DNA sequences as a fusion protein. Eukaryotic recombinant molecules may include intervening and/or untranslated sequences surrounding and/or within zona pellucida DNA sequences.

Preferred expression vectors include bacterial, yeast, and insect vectors. Particularly preferred expression vectors are those that function in E. coli or in insect cells. A most preferred E. coli vector is pGEX-2T (available from Pharmacia) which contains tac regulatory sequences and which leads to the expression of cDNA sequences as part of a fusion protein with the carboxyl terminus of glutathione-S-transferase (GST) from Schistosoma japonicum. Most preferred insect vectors are baculovirus (Autographa californica nuclear polyhedrosis virus) vectors, such as pVL1393 from InVitrogen.

Recombinant molecules useful in the present invention include, but are not limited to, zona pellucida nucleic acid sequences inserted into expression vectors capable of being expressed in bacteria, yeast, fungi, insects, animals or plants. Preferred recombinant molecules include bacterial and insect cell expression vectors containing one or more zona pellucida DNA sequences. In one embodiment, zona pellucida DNA sequence ZPDS.411 is operatively linked to bacterial expression vector pGEX-2T to form recombinant molecule pGEX2T:ZPDS.411 (see Figure 5). In another embodiment, zona pellucida DNA sequence ZPDS.1711 is operatively linked to baculovirus vector pVL1393 to form recombinant molecule pVL1393:ZPDS.1711 (see Figure 6).

The present invention also includes a method to transform prokaryotic or eukaryotic hosts with recombinant molecules, as well as the transformed hosts themselves. As discussed above, transformation can be accomplished by any process in which a recombinant molecule is inserted into a host, wherein the host may remain unicellular or may grow into a tissue or multicellular organism. Preferred hosts include bacteria, yeast, fungi, insects, insect cells, animals, animal cells, plants, and plant cells. In the practice of the present invention, the most preferred bacterial host is E. coli, and the most preferred insect cell host is Spodoptera frugiperda.

Native zona pellucida proteins are highly glycosylated and it is believed that important zona pellucida antigenic determinants are carbohydrates. Earlier studies have shown that enzymatically or chemically deglycosylated zona pellucida proteins elicit the production of antibodies with different characteristics than those induced by native zona pellucida proteins. It is as yet unknown what type of responses are elicited by totally nonglycosylated proteins, such as would be produced by bacteria. Insect cells exhibit a somewhat different glycosylation pattern than do mammalian cells with respect to sugar composition, chain length and complexity. Without being bound by theory, it is believed that carbohydrate differences may enhance mammalian immune responses to insect cell-derived recombinant antigens. Thus, in accordance with a preferred embodiment of the present invention, eukaryotic cells are

used as hosts due to their ability to glycosylate proteins. Insect cells are preferred as hosts because they are able to produce large quantities of glycosylated recombinant zona pellucida proteins.

In accordance with the present invention, a recombinant molecule containing at least one zona pellucida DNA sequence may be transformed into a host. Such a transformed host can then be cultured in an effective medium to produce recombinant protein(s) encoded by the zona pellucida DNA sequence(s). The recombinant protein(s) can subsequently be formulated into a contraceptive vaccine.

In a particular embodiment of the present invention, zona pellucida DNA sequence ZPDS.1711 is inserted into the E. coli expression vector pGEX-2T to produce recombinant molecule pGEX2T:ZPDS.1711. Recombinant molecule pGEX2T:ZPDS.1711 is transformed into E. coli, producing transformed host E. coli pGEX2T:ZPDS.1711.

In another embodiment of the present invention, zona pellucida DNA sequence ZPDS.1711 is inserted into baculovirus vector pVL1393 to produce recombinant molecule pVL1393:ZPDS.1711. Recombinant molecule pVL1393:ZPDS.1711 is transformed into Spodoptera frugiperda insect cells, producing transformed host S. frugiperda pVL1393:ZPDS.1711.

Using the techniques of recombinant DNA technology, major influences on gene expression are achieved by manipulating, for example, the number of copies of the gene within the cell, the efficiency with which those gene

copies are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of zona pellucida DNA sequences include, but are not limited to, insertion of zona pellucida DNA sequences into high-copy number plasmids, insertion of zona pellucida DNA sequences into one or more host chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Delgarno sequences), modification of zona pellucida DNA sequences to correspond to the codon usage of the host, expression of zona pellucida proteins as fusion proteins, deletion of sequences that destabilize transcripts, insertion of intervening sequences into zona pellucida DNA sequences expressed in eukaryotic cells, and use of control signals that temporally separate transformed host cell growth from zona pellucida protein production during fermentations. The economics of production may be improved by expressing more than one zona pellucida protein in a given host. The activity of the expressed zona pellucida protein may be improved by fragmenting, modifying, or derivatizing zona pellucida DNA sequences or recombinant zona pellucida proteins using standard techniques.

According to the present invention, hosts transformed with recombinant molecules containing zona pellucida

nucleic acid sequences, preferably zona pellucida DNA sequences, are cultured in an effective medium according to standard techniques. As used herein, an "effective medium" refers to any medium in which a transformed host can produce recombinant zona pellucida proteins. An effective medium is typically an aqueous medium comprising assimilable carbohydrate, nitrogen, and phosphate sources, as well as appropriate salts, minerals, metals and other nutrients, such as vitamins. The medium may comprise complex nutrients or may be a defined minimal medium. Culturing can be conducted in conventional fermentation bioreactors, which include, but are not limited to, batch, fed-batch and continuous fermenters, as well as transformed plants and animals. Preferably, a single transformed host is cultured at a time. Culturing is carried out at a temperature, pH, and oxygen content appropriate for the host.

In one embodiment, culturing comprises two steps. Hosts are initially grown in a medium effective to promote growth of the hosts. After the host has reached a desired cell density, a second medium is introduced that promotes production of recombinant zona pellucida proteins. The second medium can include a compound that induces expression of the recombinant proteins. For example, expression of a zona pellucida DNA sequence under the control of E. coli lac regulatory sequences can be induced by the addition of, for example, lactose or isopropyl-beta-D-thiogalactopyranoside (IPTG).

In one embodiment, an E. coli host transformed with at least one zona pellucida DNA sequence is grown in an effective medium at a temperature from about 32°C to about 45°C, and at a pH from about pH 6.8 to about pH 7.4.

In another embodiment, insect cell hosts transformed with at least one zona pellucida DNA sequence are cultured in an effective medium at a temperature from about 25°C to about 30°C, preferably from about 26°C to about 28°C, and at a pH from about pH 6.1 to about pH 6.5, preferably from about pH 6.3 to about pH 6.4.

Depending on the vector and host system used for production, resultant recombinant zona pellucida proteins may either (1) remain within the transformed host cell, (2) be secreted into the fermentation medium, (3) be secreted into a space between two cellular membranes, such as the periplasmic space in E. coli, or (4) be retained on the outer surface of a cell or viral membrane. Recombinant zona pellucida proteins may be recovered using a combination of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, and hydrophobic interaction chromatography.

Preferably, recombinant zona pellucida proteins are recovered in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein as a vaccine or diagnostic. A contraceptive vaccine for animals, for example, should exhibit no substantial toxicity and should be capable of

stimulating the production of antibodies in a vaccinated animal. It is within the scope of the present invention to recover recombinant zona pellucida proteins having a purity of at least about 90%.

It is within the scope of the present invention that the recombinant zona pellucida proteins and zona pellucida DNA sequences of the present invention can also be used to derive additional zona pellucida DNA sequences and zona pellucida proteins, such as full-length cDNA sequences or zona pellucida proteins and DNA sequences of other species.

Another aspect of the present invention involves the use of recombinant zona pellucida proteins to monitor the ability of a vaccinated animal to produce antibodies specific to the zona pellucida or to identify females who may be infertile because they produce antibodies that bind to their own zonae pellucidae. In one embodiment, the recombinant proteins can be labeled, for example with a radioactive, enzymatic, or fluorescent label, and mixed with a bodily fluid sample, such as a serum or urine sample, which may contain zona pellucida-specific antibodies in a medium, such as a buffer, which allows the antibodies to bind to the labeled recombinant protein. The amount of recombinant protein added to the sample should be sufficient to allow substantially all zona pellucida-specific antibodies present in the sample to bind to said recombinant protein. Complexes can be separated from unbound recombinant protein and analyzed to determine the

amount of zona pellucida-specific antibodies present in the sample.

Antibodies produced by vaccination of an animal with the recombinant proteins of the present invention can also be used as a passive contraceptive vaccine or can be used to recover zona pellucida proteins, including recombinant zona pellucida proteins, from a mixture of proteins and other contaminants.

Production and Uses of Contraceptive Vaccines

The present invention includes a safe and cost-effective contraceptive vaccine that, depending on its mode of administration, provides reversible or permanent contraception. A vaccine of the present invention contains at least one of the proteins, or portions thereof, that make up the zona pellucida. The zona pellucida protein, or portion thereof, can be recombinant, isolated from a natural source, or chemically synthesized. Vaccines of the present invention are capable of triggering the production of zona pellucida-specific antibodies that bind to the zona pellucida, thereby preventing sperm attachment. When administered to an animal that has not yet reached puberty, a vaccine of the present invention can cause disruption of ovarian function, leading to sterility. Vaccines of the present invention can be administered to any animal, including humans, in which it is efficacious. Preferred animals are mammals, which are known to have zonae pellucidae surrounding their eggs.

Due to the wide degree of structural and functional diversity among zona pellucida proteins of different species, it cannot be assumed that a vaccine based on zona pellucida proteins from one species will be as efficacious as a vaccine based on the zona pellucida proteins of a different species. However, because porcine zonae pellucidae cross-react immunologically with the zonae pellucidae of a number of other mammals, it is believed that vaccines based on porcine zona pellucida proteins will be efficacious. In addition, some studies using partially purified porcine zona pellucida proteins as a contraceptive vaccine suggest that such a vaccine may be effective in a number of animals including, but not limited to, dogs, horses, rabbits, and monkeys. However, until now, porcine zona pellucida proteins, or portions thereof, could not be produced in the amounts and purity required to develop and test a commercially feasible zona pellucida protein-based vaccine. In a preferred embodiment of the present invention, a contraceptive vaccine comprises at least one substantially pure recombinant porcine zona pellucida protein, or portion thereof.

One embodiment of the present invention is a contraceptive vaccine comprising one or more recombinant zona pellucida proteins, present in an amount effective to prevent conception. A contraceptive vaccine of the present invention is preferably produced by culturing one or more hosts transformed with one or more zona pellucida DNA sequences in a manner that allows for production of one or

more recombinant zona pellucida proteins, recovering said proteins, and combining one or more of the proteins to obtain a vaccine. A preferred contraceptive vaccine is one containing more than one recombinant zona pellucida protein. While not being bound by theory, it is believed that an advantage of using more than one recombinant zona pellucida protein is that a vaccinated animal will be exposed to a greater number of epitopes and will thus produce a group of antibodies capable of binding to a greater number of sites on the zona pellucida.

A contraceptive vaccine of the present invention can include, but is not limited to, at least a portion of one or more recombinant versions of natural porcine zona pellucida proteins selected from the group ZP1, ZP2, ZP3-alpha, ZP3-beta, ZP4, or mixtures thereof (i.e., proteins derived from recombinant DNA technology). Preferably, a vaccine of the present invention comprises at least one protein encoded by at least a portion of a zona pellucida DNA sequence selected from the group ZPDS.2500, ZPDS.1711, ZPDS.1176, ZPDS.535, ZPDS.411, ZPDS.311, ZPDS.447, or a mixture thereof.

The inventor is not aware of any reports of the successful use of one or more recombinant zona pellucida proteins to prevent conception in animals. In fact, Dean (ibid.) teaches away from the use of recombinant zona pellucida proteins derived from genetically-engineered hosts by citing a failure of a recombinant mouse ZP3-beta-

galactosidase fusion protein to elicit the production of detectable antibodies specific for native mouse ZP3.

In a preferred embodiment of the present invention, recombinant zona pellucida proteins contained in the contraceptive vaccine are glycosylated. Without being bound by theory, it is believed that glycosylated proteins may elicit the production of antibodies that are better able to recognize and to bind to the highly glycosylated proteins on a vaccinated animal's zona pellucida because antibodies raised against glycosylated proteins will be able to recognize carbohydrate as well as protein determinants. Since insects and yeast apparently do not glycosylate proteins in exactly the same manner as do mammalian cells, it is also believed that proteins glycosylated by insects or yeast may actually promote a stronger immune response in animals than would proteins glycosylated by a mammalian cell because insect- or yeast-produced glycoproteins will be more antigenic for mammals.

According to one embodiment of the present invention, the contraceptive vaccine can also include an adjuvant or a carrier to improve its efficacy. Adjuvants are typically substances that generally enhance the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, Freund's adjuvant, aluminum-based salts, calcium-based salts, silica, polynucleotides, saponin, coat proteins from viruses (such as feline panleukopenia virus), bacterial-derived preparations (such as from Mycobacterium tuberculosis), and proprietary

adjuvants such as Titermax®. A preferred adjuvant of the present invention comprises aluminum hydroxide.

Carriers are typically compounds that increase the half-life of a vaccine in a vaccinated animal. Suitable carriers include, but are not limited to, polymeric controlled-release formulations, biodegradable implants, liposomes, bacteria (such as genetically-engineered M. tuberculosis), viruses (such as genetically-engineered vaccinia virus), oils, esters, and glycols.

One aspect of the present invention includes a process for preventing conception by administering an effective dose of a contraceptive vaccine of the present invention to prevent conception. The ability of a contraceptive vaccine of the present invention to prevent conception can be reversible, or permanent, depending on the mode of administration. As used herein, the "mode of administration" includes the dose of the vaccine, the number and schedule of vaccinations, the age of the animal, and the route of administration (e.g., subcutaneous, intradermal, intravenous, nasal, oral, transdermal, and intramuscular.)

Animals which can be vaccinated according to the present invention include, but are not limited to, cats, dogs, humans, pigs, sheep, cattle, horses, burros, rabbits, elk, and deer. Preferably an animal is administered a vaccine that is derived from a species other than itself. That is, preferably the zona pellucida proteins of the vaccine are proteins, or portions thereof, that are derived

from a different species, and/or that are produced in a host of a different species.

In one embodiment, animals are vaccinated to provide reversible contraception. As used herein, "reversible contraception" is a form of contraception from which an animal can recover (i.e., it is not permanent). Reversible contraception can range in time from weeks to months to years depending on the species, vaccine dose and timing of administration. Preferably, the vaccinated animal will produce antibodies that will bind to its zona pellucida but will not sustain permanent damage to its ovaries or other components of the reproductive process. The vaccine can be administered one or more times, preferably from about one to about three times, over from an about one-month to an about three-month period. The vaccine dose (i.e., the amount of vaccine administered at one time) can range from about 25 μg to about 500 μg of recombinant protein. Preferably, a dose of from about 50 μg to about 100 μg of recombinant protein is administered. Administration can be by a variety of modes including, but not limited to, subcutaneous, intradermal, intravenous, nasal, oral, transdermal, and intramuscular routes. Preferred modes of administration include subcutaneous and intramuscular routes.

Another aspect of the present invention includes a process for sterilizing an animal by administering to the animal an effective dose of a contraceptive vaccine of the present invention capable of rendering the animal sterile.

As used herein, an effective dose to render an animal sterile is a dose which disrupts the ovaries to an extent that permanently prevents conception. Contraceptive vaccines of the present invention capable of rendering an animal sterile can contain zona pellucida proteins, or portions thereof, that are recombinant, isolated from natural sources and/or chemically synthesized. Preferably, the vaccine comprises recombinant zona pellucida proteins. The inventor is unaware of the use of any zona pellucida protein to sterilize an animal.

An animal to be sterilized is preferably administered a vaccine that will stimulate both antibody and cell mediated immunity, particularly against a vaccinated animal's ovaries. A vaccine is preferably administered one or more times before the animal reaches puberty, i.e., before the animal exhibits tolerance to proteins on its own zona pellucida. Animals which can be rendered sterile include any mammal, such as, but not limited to cats, dogs, humans, pigs, sheep, cattle, horses, burros, rabbits, elk, and deer.

In a preferred embodiment, an animal, such as a cat or dog, is first administered a contraceptive vaccine from about eight weeks to about sixteen weeks after birth, preferably from about ten weeks to about fourteen weeks after birth. The contraceptive vaccine can be administered from about once to about three times over a period of from about six weeks to about ten weeks, preferably from about

one time to about two times, over a period of from about two weeks to about three weeks.

In accordance with the present invention, the dose of the contraceptive vaccine preferably is such that only a few administrations are necessary. Preferred doses range from about 25 μg to about 500 μg and more preferably from about 50 μg to about 100 μg of protein. A contraceptive vaccine that sterilizes an animal can be administered by a variety of modes including, but not limited to, subcutaneous, intradermal, intravenous, nasal, oral, transdermal, and intramuscular routes. Preferred modes of administration include subcutaneous and intramuscular routes.

Zona pellucida proteins comprising the vaccine can be produced by culturing hosts transformed with zona pellucida DNA sequences that encode at least a portion of a zona pellucida protein that is capable of rendering an animal sterile and by recovering proteins therefrom.

In one embodiment of the present invention, a host capable of glycosylating proteins, preferably an insect cell, transformed with a zona pellucida DNA sequence such as ZPDS.1711, is cultured to produce a recombinant zona pellucida protein. A vaccine comprising the recombinant protein, either alone, or in combination with at least one other recombinant zona pellucida protein, such as a recombinant zona pellucida protein encoded by ZPDS.311 and/or by ZPDS.447, is injected into an animal one or more times over a time period of from about three weeks to about

eight weeks. The efficacy of the vaccine to prevent conception can be tested in a variety of ways including, but not limited to, detection of zona pellucida-specific antibodies in the vaccinated animal's serum, examination of the vaccinated animal's ovaries, detection of ovulation, and mating of the vaccinated animal to a male.

In another embodiment of the present invention, a bacterial host, preferably E. coli, transformed with a zona pellucida DNA sequence such as ZPDS.411, is cultured to produce a recombinant zona pellucida protein such as rZPP.137. The recombinant protein is recovered and injected into an animal, preferably a rabbit, one or more times over a time period of about seven weeks. In one embodiment, a fusion protein containing the carboxyl terminus of S. japonicum glutathione-S-transferase fused to rZPP.137 (GST-rZPP.137) is injected with an adjuvant subcutaneously and intramuscularly into a rabbit several times, with the injections being about one week apart. About one week after the final injection, serum is collected from the rabbit and analyzed for the presence of antibodies specific for porcine and/or canine zonae pellucidae. Ovaries from immunized rabbits are analyzed for abnormalities in follicular morphology and compared to ovaries from non-immunized rabbits.

In yet another embodiment, a vaccine containing more than one porcine zona pellucida protein is used to sterilize an animal. For example, young rabbits, preferably about five weeks old, were administered a

vaccine comprising solubilized porcine zona pellucida several times over about six weeks. About three to four months after the first injection, vaccinated rabbits were essentially unable to ovulate. In contrast, rabbits immunized with a control protein (one that does not induce contraception) were able to ovulate and produce fetuses.

The following experimental results are provided for purposes of illustration and are not intended to limit the scope of the invention.

Example 1: Production of rabbit antiserum to solubilized porcine zonae pellucidae.

Porcine zonae pellucidae were isolated from pig ovaries using techniques similar to those described by Dunbar et al. (Biol. Reprod. 22, 941-954, 1980). The isolated zonae pellucidae were solubilized in 0.1 mM bicarbonate buffer at pH 9.0 by heating to 68°C for 20 min. Approximately 1000 zonae pellucidae (about 40 μg of protein) in 100 μl (microliters) were mixed with 100 μl of Freund's complete adjuvant and injected into multiple subcutaneous and intramuscular sites of a rabbit. Four, eight and twelve weeks later, the rabbit was boosted with the same dose of solubilized zonae pellucidae in Freund's incomplete adjuvant. Serum was collected one week after the final immunization and was shown to contain antibodies specific for solubilized porcine zona pellucida by its ability to (a) react with intact porcine zonae pellucidae using an immunofluorescence assay, (b) react with zonae

pellucidae present in pig ovary sections using an immunohistochemistry assay, and (c) react with solubilized zonae pellucidae using an immunoblot-based assay. This serum was subsequently used to screen a porcine ovary cDNA expression library.

Example 2: Isolation of clones containing zona pellucida DNA sequences.

Total RNA was isolated from a porcine ovary by a guanidinium/cesium chloride method described by Glisin et al. (Biochemistry 13, 2633-2638, 1974) and Ullrich et al. (Science 196, 1313-1319, 1977). Polyadenylated RNA was isolated from total RNA by chromatography on oligo-dT-cellulose as described by H. Aviv & P. Leder (Proc. Natl. Acad. Sci. 69, 1408, 1972) and stored at -70°C as an ethanol precipitate. Double stranded cDNA was produced using a kit available from Life Technologies Inc. (GIBCO BRL) of Gaithersburg, MD. The resultant cDNA was treated with EcoRI methylase to methylate EcoRI restriction sites within cDNA fragments. EcoRI linkers were then added to the ends of the cDNA, and the linkered cDNA ligated into E. coli bacteriophage lambda phage gt11 arms, using techniques described by Sambrook et al. (ibid.). cDNA ligated into the lambda phage arms was packaged into phage particles using a Gigapack II Plus packaging extract, available from Stratagene of La Jolla, CA. The resulting cDNA expression library was titered by plaque assay on E. coli Y1090 and

stored in phage buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris, pH 7.5, 0.01% gelatin) with chloroform at 4°C.

Primary screening of the cDNA expression library was begun by incubating approximately 50,000 plaque forming units of cDNA-containing phage with E. coli Y1090 per 150 x 10 mm petri dish containing LB-agar solidified growth medium (10 g Bacto-Tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, in 1 liter water). Plates were incubated at 42°C for 3.5 hours, then transferred to a 37°C incubator for 4.5 hours. The agar surfaces were overlaid with nylon filters (e.g., Zeta-Probe which is available from Biorad of Richmond, CA) that had been soaked in 10 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and partially dried. Growth was continued overnight.

Filters were marked with a 30 gauge needle at asymmetric points around their periphery, removed from the agar-containing plates, washed briefly at room temperature in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20) and then submitted to two 30 min. washes in TBST at room temperature. Washed filters were incubated in blocking buffer (TBST containing 10% bovine serum and 1.25% nonfat dry milk) for 3 to 4 hours, washed twice for 30 min. each in TBST, and incubated with an absorbed and diluted primary antibody solution for 3 to 4 hours, all at room temperature. The absorbed and diluted primary antibody solution was prepared by mixing rabbit antiserum containing antibodies specific for a solubilized porcine zona pellucida (prepared as described in Example 1) with TBST

containing 1% bovine serum and a 1:1000 dilution of Y1090 extract for 3 hours at 4°C, after which the antiserum was diluted to a final dilution of 1:500 in TBST. After incubation of the filters in the absorbed and diluted primary antibody solution, the filters were submitted to three 10-min. washes with TBST at room temperature and incubated for 45 min. at room temperature with an anti-rabbit IgG antibody to which alkaline phosphatase was attached, available from Sigma, of St. Louis, MO. Filters were then washed at room temperature twice with TBST for 10 min. each, and once with TBS (TBST without 0.1% Tween) for 5 min. The filters were then immersed in NBT-BCIP substrate. NBT-BCIP substrate was prepared just before use in a buffer consisting of 100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$. To 30 ml of this buffer were added 990 μl (microliters) of 25 mg nitroblue tetrazolium (NBT, from Sigma) per ml water and 99 μl of 20 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP, from Sigma) per ml 2,4-dimethylformamide.

Filters showing positive signals (i.e., purple plaques) on primary screening were aligned with their matching plates and the area around the positive signal was removed using the large end of a pasteur pipet. The plug of agar was placed into 1 ml of phage buffer and the phage were allowed to diffuse out of the agar for 2 to 3 hr at room temperature. These phage were rescreened using the same protocol as described above until single plaque isolates were obtained that bound to rabbit antiserum

containing antibodies specific for solubilized porcine zonae pellucidae. Approximately 1 in 300,000 phage screened contained a cDNA sequence encoding a protein that bound to antibodies specific for porcine zonae pellucidae.

Single plaque isolates were amplified and DNA was purified therefrom to identify the size and sequence of the DNA insert. Phage DNA was digested with EcoRI, and the resulting cDNA insert was ligated into EcoRI-restricted Bluescript KS plasmids, available from Stratagene of La Jolla, CA. Sequencing was performed using Sequenase kits, available from US Biochemical Corp., using techniques recommended by the manufacturer. Intelligenetics Inc.'s PC/GENE program and software written in-house were used to align data from DNA sequencing runs, to generate restriction maps, to translate DNA sequences into protein sequence, and to search for potential glycosylation sites.

One of the single isolates was found to have a cDNA fragment of approximately 2500 base pairs (bp) in length which was called zona pellucida DNA sequence 2500, or ZPDS.2500. ZPDS.2500 is composed of 3 EcoRI restriction fragments of about 535 bp, about 1176 bp, and about 800 bp, organized as diagrammed in Figure 1. The DNA sequence of the about 535-bp (ZPDS.535) fragment joined to the about 1176-bp (ZPDS.1176) fragment, called ZPDS.1711, is presented in Figure 2. Also shown is the deduced 305-amino acid sequence, called rZPP.305, which represents the putative carboxyl terminal portion of a porcine zona pellucida protein. rZPP.305 exhibits a putative N-linked

glycosylation site at amino acid 288. DNA sequence analysis of the third EcoRI fragment of about 800 bp indicated that this fragment contains a polyadenylation site and at least a portion of a polyadenylation tail. Immunoprecipitation data (described in Example 5) suggest that rZPP.305 corresponds to a portion of porcine zona pellucida protein ZP2, which has a reported deglycosylated molecular weight of from about 52 kD to about 56 kD (Hedrick and Wardrip, 1987, ibid.), corresponding to about 460 amino acids. The PALIGN program was used to compare protein homologies between rZPP.305 and published deduced protein sequences of other zona pellucida proteins. rZPP.305 showed no similarity to mouse ZP2, mouse ZP3, hamster ZP3, human ZP3, or a rabbit 55-kD deglycosylated zona pellucida protein. rZPP.305 was also compared to 20,024 other protein sequences in the Swiss-Prot data base; no similarities above 4% were observed.

A second single isolate was found to contain a cDNA fragment of approximately 311 bp in length, called ZPDS.311. The DNA sequence of ZPDS.311 is presented in Figure 3 as is the deduced 103-amino acid sequence, called rZPP.103. The protein sequence exhibits a putative N-linked glycosylation site at amino acid 43. rZPP.103 does not share significant sequence homology with any other zona pellucida protein.

A third single isolate was found to contain a cDNA fragment of approximately 447 bp in length, called ZPDS.447. The DNA sequence of ZPDS.447 is presented in

Figure 4 as is the deduced 149-amino acid sequence, called rZPP.149. The protein sequence exhibits putative N-linked glycosylation sites at amino acids 108 and 143. rZPP.149 does not share significant sequence homology with any other zona pellucida protein.

Example 3: Expression of a zona pellucida DNA sequence in bacterial cells.

A 411-bp HincII/EcoRI fragment of ZPDS.535, called ZPDS.411 (depicted in Figure 1) was ligated into the E. coli expression vector pGEX-2T (Smith and Johnson, 1988; available from Pharmacia) which had been digested with SmaI and EcoRI, using standard techniques as described in Sambrook et al. (ibid.). (HincII and SmaI both generate blunt ends.) The resultant recombinant molecule was called pGEX2T:ZPDS.411, and is shown in Figure 5. ZPDS.411 was inserted into the pGEX-2T expression vector in such a way that cells transformed with the recombinant molecule pGEX2T:ZPDS.411 would express ZPDS.411 as a fusion protein containing the carboxyl terminus of Schistosoma japonicum glutathione-S-transferase (GST) at the amino terminus and a recombinant zona pellucida protein of 137 amino acids (rZPP.137) encoded by ZPDS.411 at the carboxyl terminus, yielding GST-rZPP.137. Expression of GST-rZPP.137 was under the control of the IPTG-inducible tac promoter.

pGEX2T:ZPDS.411 was transformed into E. coli strain DH5-alpha to obtain transformed host E. coli pGEX2T:ZPDS.411, using techniques as described in Sambrook

et al. (ibid.). To express the GST-rZPP.137 fusion protein, the transformed E. coli host was grown overnight in 50 ml of an aqueous fermentation medium comprising LB at 37°C, diluted 1:10 into 500 ml fresh LB at 37°C and grown to mid-log phase (OD_{600} of 0.6-1.0). IPTG was added to a final concentration of 1 mM and incubation continued for an additional 5 hr. Bacteria were collected by centrifugation and sonicated briefly. The cell sonicate was mixed with an agarose resin to which glutathione was covalently attached (e.g., Glutathione Sepharose 4B from Pharmacia) in binding buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) to allow the GST portion of the GST-rZPP.137 fusion protein to bind to the glutathione on the resin, whereas other materials in the suspension did not bind. GST-rZPP.137 was subsequently eluted from the resin using a glutathione-containing solution (5 mM glutathione, 50 mM Tris, pH 8.0).

GST-rZPP.137 prepared in this manner is ready for use in a contraceptive vaccine.

Example 4: Expression of a zona pellucida DNA sequence in insect cells.

Zona pellucida DNA sequence ZPDS.1711 was first ligated into the multicloning site of Stratagene's pBLUESCRIPT II KS(-) plasmid vector to form PZP31. In order to transfer ZPDS.1711 into baculovirus expression vector pVL1393 (available from InVitrogen of San Diego, CA), PZP31 was submitted to a partial EcoRI digest and a KpnI digest (there is a KpnI site within the vector's

multicloning site). The EcoRI/KpnI fragment containing ZPDS.1711 was mixed with both the BamHI/EcoRI oligoadapter

5'GATCCAAATATGCCGG
GTTTATACGGCCTAA

that contains consensus baculovirus translation initiation sequences and a pVL1393 vector which had been cleaved with BamHI and KpnI (see Figure 6) to obtain recombinant molecule pVL1393:ZPDS.1711. ZPDS.1711 was inserted into pVL1393 such that expression of ZPDS.1711 was under the control of the regulatory region of the baculovirus polyhedrin gene and such that rZPP.305 does not contain any baculovirus amino acids.

pVL1393:ZPDS.1711 was introduced into Spodoptera frugiperda insect cells coincidentally with wild type baculovirus DNA, using techniques similar to those described by InVitrogen. Resulting recombinant baculovirus were identified by standard hybridization methods. The recombinant baculovirus were shown by an immunoblotting experiment to encode a protein of the size expected for rZPP.305 that bound to antibodies specific for solubilized porcine zonae pellucidae (prepared as in Example 1). To produce large quantities of rZPP.305, S. frugiperda infected with the recombinant baculovirus (i.e., transformed host S. frugiperda pVL1393:ZPDS.1711) was grown in Grace's insect medium at about 27°C. rZPP.305 protein was purified from infected cell lysates by differential centrifugation and conventional column chromatographic techniques or by polyacrylamide gel electrophoresis.

rZPP.305 prepared in this manner is ready for use in a contraceptive vaccine.

Example 5: Use of a recombinant zona pellucida protein as a vaccine.

GST-rZPP.137, produced as described in Example 4, was injected into a rabbit using the following protocol. One mg of fusion protein in a volume of 500 μl was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously and intramuscularly. Booster injections of the same dose in Freund's incomplete adjuvant were given every 7 days for 6 weeks.

Antiserum (GST-rZPP.137 antiserum) was collected one week after the final immunization and was shown to react with porcine zona pellucida by immunofluorescent staining of intact porcine zonae pellucidae. The fluorescence observed using GST-rZPP.137 antiserum was not as strong as that seen with antiserum obtained from an animal immunized with solubilized porcine zonae pellucidae as described in Example 1 (pSZP antiserum), but was much stronger than that seen with serum from a non-immunized animal (non-immune serum). Immunoperoxidase staining of sections of porcine ovary exposed to GST-rZPP.137 antiserum indicated that antibodies in the antiserum bound specifically to the zona pellucida. Similar experiments indicated that GST-rZPP.137 antiserum also specifically recognized and bound to canine zonae pellucidae.

In an immunoprecipitation assay, solubilized porcine zona pellucida proteins were labeled with ^{125}I-iodine and incubated with GST-rZPP.137 antiserum as well as with the following controls: non-immune rabbit serum, serum from a rabbit immunized with an E. coli lysate plus Freund's adjuvant (E. coli lysate serum), and antiserum against solubilized porcine zonae pellucidae (pSZP antiserum). The resultant complexes were isolated using protein A sepharose, submitted to one-dimensional polyacrylamide gel electrophoresis (PAGE), and analyzed by autoradiography, using standard techniques as described by Sambrook et al. (ibid.). No radioactive signal was observed in samples in which ^{125}I-zona pellucida proteins were mixed with either non-immune rabbit serum or with E. coli lysate serum. Electrophoresis of complexes formed between ^{125}I-zona pellucida proteins and pSZP antiserum resolved as a broad band of radioactivity, indicative of overlapping zona pellucida glycoproteins. A distinct, but much more narrow band was observed in the sample containing ^{125}I-zona pellucida proteins complexed with GST-rZPP.137 antiserum, indicating the antibodies likely reacted with a single class of zona pellucida glycoproteins.

Complexes formed between ^{125}I-zona pellucida proteins and GST-rZPP.137 antiserum were also submitted to two-dimensional PAGE. GST-rZPP.137 antiserum complexed with a single species of ^{125}I-zona pellucida protein, which appeared most likely to be ZP2.

Example 6: Use of solubilized porcine zona pellucida as a vaccine to sterilize rabbits.

Porcine zonae pellucidae were isolated and solubilized as described in Example 1. A contraceptive vaccine dose comprising approximately 1000 zonae pellucidae (about 40 μg of protein) per 100 μl of Freund's complete adjuvant was prepared. A vaccine dose was injected into each of five female rabbits, which were about five to about six weeks old, into multiple subcutaneous and intramuscular sites. Two and four weeks later, the rabbits were boosted with the same dose of solubilized zonae pellucidae in Freund's incomplete adjuvant. Concomitant with this immunization protocol, five additional female rabbits of the same age were administered a control vaccine containing 500 μg of Schistosoma japonicum glutathione-S-transferase (GST) in Freund's adjuvant, using the same immunization protocol.

Each of the ten rabbits was injected with 10 μg of gonadotropin-releasing hormone and artificially inseminated 134 days after the first immunization. Two weeks later, the rabbits were killed and analyzed for number of corpora lutea (# CLs, indicative of number of ovulations) and number of implantation sites (# Imp, indicative of number of fetuses). The results are shown in Table 1.

Table 1

Sterilization of Young Rabbits

Control Rabbits			Zona pellucida Treated Rabbits		
Rabbit	#CLs	#Imp	Rabbit	#CLs	#Imp
2	0	0	4	0	0
7	9	8	9	0	0
6	9	5	12	0	0
11	2	0	13	0	0
15	12	0	16	9	1
mean:	6.4	2.6		1.8	0.2

Four of the five rabbits administered the zona pellucida protein-containing vaccine demonstrated no ovulations, whereas four of the five control rabbits exhibited multiple ovulations. It is believed that the low number of implantations per corpora lutea may be due to the young age of the rabbits. In addition, the ovaries of the rabbits administered the zona pellucida protein-containing vaccine were quite small and immature, without antral follicles, compared to the control rabbits.

These results strongly support the preferred use of more than one porcine zona pellucida protein to sterilize animals.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims:

What is claimed is:

1. A recombinant zona pellucida protein capable of preventing conception, wherein said protein is capable of stimulating the production of zona pellucida-specific antibodies when administered to an animal.

2. The protein of Claim 1, wherein said protein comprises porcine zona pellucida sequences.

3. The protein of Claim 1, wherein said protein is capable of binding to antibodies specific for a solubilized porcine zona pellucida.

4. The protein of Claim 1, wherein said protein is selected from the group comprising recombinant versions of natural porcine zona pellucida proteins ZP1, ZP2, ZP3-alpha, ZP3-beta, and ZP4.

5. The protein of Claim 1, wherein said protein is encoded by a DNA sequence selected from the group comprising ZPDS.2500, ZPDS.1711, ZPDS.1176, ZPDS.535, ZPDS.411, ZPDS.311, and ZPDS.447.

6. The protein of Claim 1, wherein one or more amino acids is added to, deleted from or substituted into said protein without substantially interfering with said protein's ability to prevent conception.

7. The protein of Claim 1, wherein said protein is substantially pure.

8. The protein of Claim 1, wherein said protein is glycosylated.

9. The glycosylated protein of Claim 8, wherein said glycosylated protein is more efficacious than an essentially deglycosylated form of said protein.

10. The protein of Claim 1, wherein said protein is capable of sterilizing an animal when administered to said animal in an effective manner.

11. A recombinant porcine zona pellucida protein, wherein said protein is produced by a process comprising:

(a) culturing in an effective medium a host transformed with a zona pellucida DNA sequence in a manner such that said host is capable of expressing said sequence as a protein; and

(b) recovering said protein.

12. The protein of Claim 11, wherein said host is selected from the group comprising bacteria, yeast, fungi, insect cells, animal cells, plant cells, insects, animals, and plants.

13. The protein of Claim 11, wherein said host comprises a cell capable of glycosylating said recombinant protein.

14. The protein of Claim 11, wherein said host is of the genus Spodoptera.

15. The protein of Claim 11, wherein said host comprises Escherichia coli.

16. The protein of Claim 11, wherein said zona pellucida DNA sequence is operatively linked to an expression vector.

17. The protein of Claim 16, wherein said expression vector is selected from the group comprising bacterial vectors, yeast vectors, fungal vectors, animal vectors, insect vectors, and plant vectors.

18. The protein of Claim 16, wherein said expression vector comprises a baculovirus vector.

19. A process for using at least one recombinant zona pellucida protein to produce zona pellucida-specific antibodies in an animal comprising administering to said animal an effective amount of said protein to produce said antibodies.

20. The process of Claim 19 further comprising recovering antibodies produced thereby.

21. Zona pellucida-specific antibodies produced in accordance with the process set forth in Claim 19.

22. A process for using zona pellucida-specific antibodies produced in accordance with Claim 20 to prevent conception, comprising administering to an animal an effective amount of said antibodies to prevent conception.

23. A process for determining infertility in a female animal, comprising:

(a) contacting a recombinant zona pellucida protein with a bodily fluid sample obtained from said female animal under conditions such that substantially all zona pellucida-specific antibodies present in said bodily fluid form a complex with said recombinant protein; and

(b) measuring the amount of complex formed thereby.

24. The process of Claim 23, wherein said process comprises determining the effectiveness of a zona pellucida-based contraceptive vaccine administered to said animal.

25. A process for producing a recombinant zona pellucida protein comprising:

(a) isolating from a pig a zona pellucida DNA sequence comprising to at least a portion of a porcine zona pellucida protein gene, wherein said portion encodes a protein capable of preventing conception;

(b) producing a recombinant molecule comprising said zona pellucida DNA sequence operatively linked to an expression vector;

(c) transforming a host with said recombinant molecule;

(d) culturing said transformed host in an effective medium to produce said recombinant zona pellucida protein; and

(e) recovering said recombinant protein.

26. The process of Claim 25, wherein said host is capable of glycosylating said recombinant protein.

27. A contraceptive vaccine comprising an amount of one or more recombinant zona pellucida proteins effective to prevent conception when administered to an animal.

28. A contraceptive vaccine of Claim 27, wherein said prevention of conception is permanent.

29. A contraceptive vaccine of Claim 27, wherein said protein comprises porcine zona pellucida sequences.

30. A contraceptive vaccine of Claim 27, wherein said vaccine comprises at least one protein selected from the group comprising recombinant versions of natural porcine zona pellucida proteins ZP1, ZP2, ZP3-alpha, ZP3-beta, and ZP4.

31. A contraceptive vaccine of Claim 27, further comprising an adjuvant or a carrier.

32. A contraceptive vaccine of Claim 27, wherein said protein is glycosylated in a manner such that said protein is capable of producing zona pellucida-specific antibodies when administered to an animal.

33. A contraceptive vaccine of Claim 27, wherein said protein is encoded by a zona pellucida DNA sequence selected from the group comprising ZPDS.2500, ZPDS.1711, ZPDS.1176, ZPDS.535, ZPDS.411, ZPDS.311, and ZPDS.447.

34. A contraceptive vaccine comprising at least one zona pellucida protein that when administered to an animal is capable of rendering said animal sterile.

35. The contraceptive vaccine of Claim 34, wherein one or more amino acids is added to, deleted from or substituted into said protein without substantially interfering with said vaccine's capability of sterilizing an animal.

36. The contraceptive vaccine of Claim 34, wherein at least one of said proteins is recombinant.

37. A process for using a contraceptive vaccine to prevent conception, wherein said vaccine comprises at least one recombinant zona pellucida protein, said process comprising administering to an animal an effective dose of said vaccine to prevent conception.

38. The process of Claim 37, wherein said protein comprises porcine zona pellucida sequences.

39. The process of Claim 37, wherein said animal is selected from the group comprising humans, cats, dogs, pigs, sheep, cattle, horses, burros, rabbits, elk, and deer.

40. The process of Claim 37, wherein said step of administering comprises administering to said animal at least about two doses of said vaccine, wherein each dose comprises from about 25 μg to about 500 μg of said protein.

41. A process for rendering an animal sterile, comprising administering to said animal a contraceptive vaccine having at least one zona pellucida protein in an amount effective to sterilize said animal.

42. The process of Claim 41, wherein said animal has not yet reached puberty.

43. The process of Claim 41, wherein one or more amino acids is added to, deleted from or substituted into said protein without substantially interfering with said protein's ability to render an animal sterile.

44. The process of Claim 41, wherein said protein comprises porcine zona pellucida sequences.

45. The process of Claim 41, wherein said animal is selected from the group comprising humans, cats, dogs, pigs, sheep, cattle, horses, burros, rabbits, elk, and deer.

46. The process of Claim 41, wherein said step of administering comprises administering to said animal at least about two doses of said vaccine, wherein each dose comprises from about 25 μg to about 500 μg of said protein.

47. The process of Claim 41, wherein said protein is recombinant.

48. A process for sterilizing an animal comprising:

(a) isolating from a mammal a zona pellucida DNA sequence;

(b) producing a recombinant molecule comprising said zona pellucida DNA sequence operatively linked to an expression vector;

(c) transforming a host with said recombinant molecule;

(d) culturing said transformed host in an effective medium to produce a recombinant protein;

(e) recovering said protein in substantially pure form;

(f) combining one or more of said proteins to obtain a contraceptive vaccine; and

(g) administering said vaccine to said animal prior to puberty in a manner effective to sterilize said animal.

49. The process of Claim 48, wherein said host is capable of glycosylating said protein.

50. An isolated nucleic acid sequence, wherein said sequence corresponds to at least a portion of a porcine zona pellucida gene.

51. A nucleic acid sequence of Claim 50, wherein said sequence comprises a zona pellucida DNA sequence.

52. A nucleic acid sequence of Claim 50, wherein said sequence is complementary DNA.

53. A nucleic acid sequence of Claim 50, wherein said sequence encodes at least a portion of porcine zona pellucida protein ZP1, ZP2, ZP3-alpha, ZP3-beta, or ZP4.

54. A nucleic acid sequence of Claim 50, wherein said sequence is isolated by a process comprising:

(a) preparing a porcine ovary complementary DNA expression library;

(b) culturing said library under conditions that promote production of proteins encoded by said complementary DNA;

(c) contacting said cultured library with antibodies specific for a solubilized porcine zona pellucida; and

(d) selecting a colony that contains a DNA sequence encoding a protein capable of binding to at least one of said antibodies.

55. A nucleic acid sequence of Claim 50, wherein said sequence is selected from the group comprising ZPDS.2500, ZPDS.1711, ZPDS.1176, ZPDS.535, ZPDS.411, ZPDS.311, and ZPDS.447.

56. A recombinant molecule comprising a nucleic acid sequence as set forth in Claim 50, operatively linked to an expression vector.

57. A host transformed with the recombinant molecule as set forth in Claim 56, and progeny thereof.

58. A host transformed with a nucleic acid sequence as set forth in Claim 50 in a manner such that the host is capable of expressing said sequence, and progeny thereof.

59. A process of using antibodies specific for a solubilized porcine zona pellucida to isolate a zona pellucida DNA sequence comprising:

(a) preparing a mammalian ovary complementary DNA expression library;

(b) culturing said library under conditions that promote production of proteins encoded by said complementary DNA;

(c) contacting said cultured library with said antibodies; and

(d) selecting a colony that contains a DNA sequence encoding a protein capable of binding to at least one of said antibodies.

60. The process of Claim 59, wherein said zona pellucida DNA sequence is selected from the group comprising ZPDS.2500, ZPDS.1711, ZPDS.1176, ZPDS.535, ZPDS.411, ZPDS.311, and ZPDS.447.

Relative Locations of Zona Pellucida DNA Sequences
ZPDS. 1711, ZPDS. 535. ZPDS. 411, and ZPDS. 1176 on ZPDS. 2500

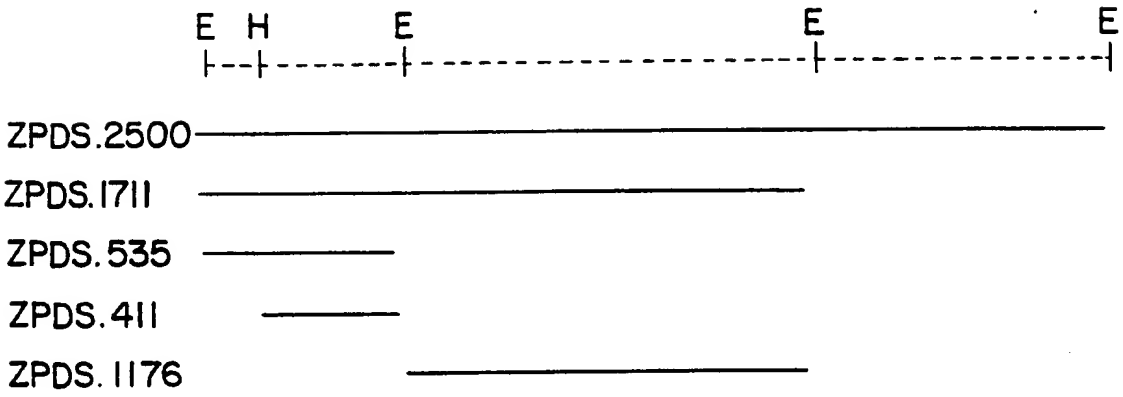


FIG. 1

FIG. 2A

DNA and Deduced Amino Acid Sequence of ZPDS.1711

```
                10                  20                  30                  40
(LINKER)        |                   |                   |                   |
GAATTCCCT GCG GCC GAC TCG GAG GCT TCC GCG GCT TCT GCG GCA CGG
          Pro Gly Gly Ser Arg Gln Lys Gln Arg Arg Lys Ser Arg

50                  60                  70                  80                  90
|                   |                   |                   |                   |
ACG GGA AGT GAG GCG GCC GGG CCG GGC GGC TCG CGG CAG AAA CAG AGG
Cys Gly Thr Ala Glu Phe Pro Ala Ala Asp Ser Glu Ala Ser Ala Ala

  100                 110                 120                 130                 140
  |                   |                   |                   |                   |
CGG AAG AGT CGC TGC GGC ACA GCG TCG TCG ACC CGG GTG ACC GAG AGT
Ser Ala Ala Arg Thr Gly Ser Glu Ser Ser Thr Arg Val Thr Glu Ser

    150                 160                 170                 180                 190
    |                   |                   |                   |                   |
GCT GAG CGC CGA GGG GAC TCG CCC GCT CGG AAG CCG GAT CCC GAG CCG
Ala Glu Arg Arg Gly Asp Ser Pro Ala Arg Lys Pro Asp Pro Glu Pro

        200                 210                 220                 230                 240
        |                   |                   |                   |                   |
GGC AGG ATG GAT CAT CAC CAG CCT GGG ACT GGT CGC TAC CAG GTG CTT
Gly Arg Met Asp His His Gln Pro Gly Thr Gly Arg Tyr Gln Val Leu
```

FIG. 2B

```
                250        260        270          280
CAT AAT GAA GAG GAT AAT TCA GAA TCA TCT GCA GTA GAG CAG CAT CAA
His Asn Glu Glu Asp Asn Ser Glu Ser Ser Ala Val Glu Gln His Gln

290         300         310        320         330
CTT CAG ACC CAG TAC CAC AGA CCA TGC AGG CTG CTT CTT CAG CAT CAG
Leu Gln Thr Gln Tyr His Arg Pro Cys Arg Leu Leu Leu Gln His Gln

    340        350        360          370        380
GAC TTG AGG CTG ACT CTT CTC CTC CAC CTT ATA GTA GTA TTA CCG TGG
Asp Leu Arg Leu Thr Leu Leu Leu His Leu Ile Val Val Leu Pro Trp

    390         400        410         420        430
AAG TAC CTA CAA CTT CAG ATA CAG AAG TTT ACA GTG ACT TTT ATC CTG
Lys Tyr Leu Gln Leu Gln Ile Gln Lys Phe Thr Val Thr Phe Ile Leu

        440        450         460        470        480
TGC CAC CTC CCT ACA GTG TTG CTA CCT CCC ATT CCT ACG TAT GAT GAA
Cys His Leu Pro Thr Val Leu Leu Pro Pro Ile Pro Thr Tyr Asp Glu
```

FIG. 2C

```
                490            500            510             520
                 |              |              |               |
GCT GAG AAG GCC AAA GCT CGT GCC ATA GCA GCC GCA GCA GCA GAA ACA
Ala Glu Lys Ala Lys Ala Arg Ala Ile Ala Ala Ala Ala Ala Glu Thr

530            540             550            560            570
 |              |               |              |              |
TCT CAA AGA ATT CAG GAG GAA GAA TGT CCA CCA AGA GAT GAC TTC AGT
Ser Gln Arg Ile Gln Glu Glu Glu Cys Pro Pro Arg Asp Asp Phe Ser

    580            590            600             610            620
     |              |              |               |              |
GAT GCA GAC CAG CTC AGA GTG GGT AAT GAT GGG ATT TTC ATG CTG GCA
Asp Ala Asp Gln Leu Arg Val Gly Asn Asp Gly Ile Phe Met Leu Ala

    630             640            650             660             670
     |               |              |               |               |
TTT TTC ATG GCA TTT ATT TTC AAC TGG CTT GGA TTT TGT TTA TCT TTC
Phe Phe Met Ala Phe Ile Phe Asn Trp Leu Gly Phe Cys Leu Ser Phe

        680            690             700            710            720
         |              |               |              |              |
TGT ATC ACC AAT ACC ATA GCT GGA AGG TAT GGT GCC ATC TGT GGA TTT
Cys Ile Thr Asn Thr Ile Ala Gly Arg Tyr Gly Ala Ile Cys Gly Phe
```

FIG. 2D

```
                   730                 740                 750                 760
                    |                   |                   |                   |
GGC CTT TCA TTG ATC AAG TGG ATT CTT ATT GTC AGG TTT TCT GAT TAT
Gly Leu Ser Leu Ile Lys Trp Ile Leu Ile Val Arg Phe Ser Asp Tyr

770             780                   790                 800                 810
 |               |                     |                   |                   |
TTT ACT GGA TAT TTC AAT GGA CAG TAT TGG CTT TGG TGG ATA TTT CTT
Phe Thr Gly Tyr Phe Asn Gly Gln Tyr Trp Leu Trp Trp Ile Phe Leu

    820                 830                 840                   850                 860
     |                   |                   |                     |                   |
GTA CTT GGC CTG CTT CTT TTC TTC AGA GGA TTT GTT AAT TAC CTG AAA
Val Leu Gly Leu Leu Leu Phe Phe Arg Gly Phe Val Asn Tyr Leu Lys

        870                   880                 890                 900                   910
         |                     |                   |                   |                     |
GTC AGA AAC ATG TCT GAA AGT ATG GCA GCT GCT CAT AGA ACA AGA TAT
Val Arg Asn Met Ser Glu Ser Met Ala Ala Ala His Arg Thr Arg Tyr

            920                 930                 940                 950                 960
             |                   |                   |                   |                   |
TTC TTC TTA TTA TAGAGACTGC ATCGACCAGA CATTCCTTTC TTATACCAAT
Phe Phe Leu Leu---
```

FIG. 2E

```
970        980        990        1000       1010       1020
GTGAAATTTC CAGATCATCT GTAAATCTGC AACTTTAATA GGATAGAACA CTACTAAAAA

1030       1040       1050       1060       1070       1080
CGGAAGACAA ATTAGTGAAG AAAAGATAGA GCTTCAAAAT TGAATGGCAG AGTGGTTTAT

1090       1100       1110       1120       1130       1140
GCTTAAAAGC CATTTCTGTT CATTCTCTTA ATATTTCATT TGCTTTGCGC ATTTGCATAT

1150       1160       1170       1180       1190       1200
GTGCCCATTT AAAAGATTTG CATATTCTTG AAAGAACCA GAAACCGTAA AGTTGTAGCA

1210       1220       1230       1240       1250       1260
GTAAAGTTTC GTCATATTTG ATTAGTGTTT GATATAATTG AAAGAGTTGA GTGATAAACA

1270       1280       1290       1300       1310       1320
GTCTTCCAGC ATGTAAATGT GATTGACTTC TGACCTGACA TTTAGTATAA TAAAAATGAA

1330       1340       1350       1360       1370       1380
ATGATTAACC ATGTCAAATG CTTTAGTTTC TGGCTCTTAG ATTTATCTAG TAACTCTGCA
```

FIG. 2F

1390	1400	1410	1420	1430	1440
CATCCTTTGT	TATATATAAA	ATTTTTTGAA	ACTTGCAGTG	CTGATTATTA	GAAAGGCTTT
1450	1460	1470	1480	1490	1500
GTCAGATTTT	TGAACATGAT	ATTTACATCA	TTATTTAGCA	AAACGTTATG	TAAATAACCA
1510	1520	1530	1540	1550	1560
TGCATAAATT	ACTTTCTGCA	TTGTTTTCTT	AGAAATTGTG	TCTAGATATC	TTTTCATTAA
1570	1580	1590	1600	1610	1620
TTTTAAATTA	AGTGGACTTA	ATATATATAG	AAAATTTTGC	CGTCAAAGAT	AGTTTGTTTT
1630	1640	1650	1660	1670	1680
AGGACAAATT	TTAAGAAAAA	TGTGGGTATT	CCTCATGTCC	TTTGTAAGAA	AATCCTTTTT
1690	1700	1710			
TTTTTTAAAG	GGACATGCCA	GTTTTAGGAA	TTC		

FIG. 3

DNA and Deduced Amino Acid Sequence of ZPDS.311

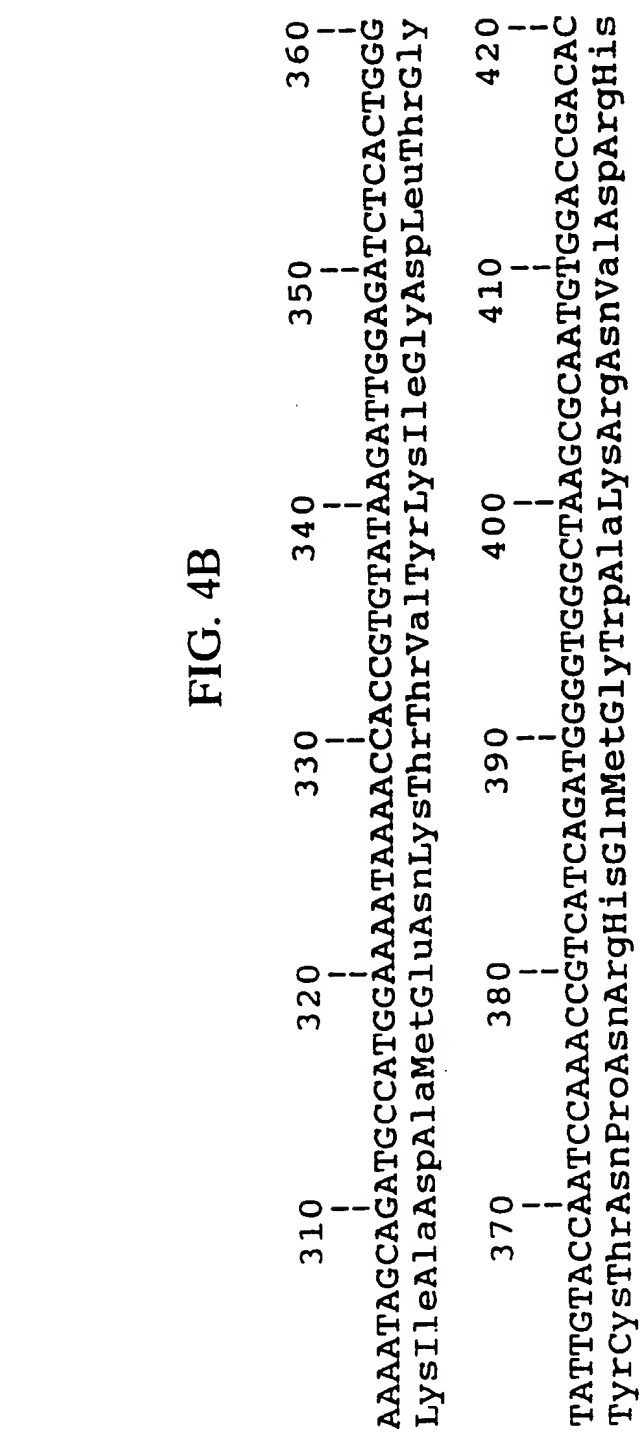
```
          10         20         30         40         50         60
          |          |          |          |          |          |
GAATTCCGGGCCGTTCCGAAGGACGGGCGATGGCCTCCGTTGCCCTCAGCCGATCGAAAGGGAGTCGGG
GluPheArgAlaValProLysAspGlyArgTrpProProLeuProSerAlaAspArgLysGlyValGly

70         80         90         100        110        120        130
|          |          |          |          |          |          |
TTCAGATCCCCGAATCCGGAGTGGCGGAGATGGGCGCCGCGAGGCGTCCAGTGCGGTAACGCAACCGAT
PheArgSerProAsnProGluTrpArgArgTrpAlaProArgGlyValGlnCysGlyAsnAlaThrAsp

140        150        160        170        180        190        200
|          |          |          |          |          |          |
CCCGGAGAAGCCGGCGGGAGCCCCGGGGAGAGTTCTCTTTTCTTTGTGAAGGGCAGGGCGCCCTGGAAT
ProGlyGluAlaGlyGlySerProGlyGluSerSerLeuPhePheValLysGlyArgAlaProTrpAsn

210        220        230        240        250        260        270
|          |          |          |          |          |          |
GGGTTCGCCCCGAGAGAGGGGCCCGTGCCTTGGAAAGCGTCGCGGTTCCGGCGGCGTCCGGTGAGCTCT
GlyPheAlaProArgGluGlyProValProTrpLysAlaSerArgPheArgArgArgProValSerSer

280        290        300        310
|          |          |          |
CGCTGGCCCTTGAAAATCCGGGGGAGCCGGAATTC
ArgTrpProLeuLysIleArgGlySerArgAsn
```

FIG. 4A

DNA and Deduced Amino Acid Sequence of ZPDS.447

```
                10        20        30        40        50        60
                |---------|---------|---------|---------|---------|---
GAATTCCGGAAGAAGAAGGGGGGCATATACAGTCAGGAGCAGGCAGCCAAAGCTGTC
GluPheArgLysLysLysGlyGlyIleTyrSerGlnGluGlnAlaAlaLysAlaVal

                70        80        90        100       110       120
                |---------|---------|---------|---------|---------|---
CTGNCTGACTGGGTGAGTGGGAAGATCAGCTTCTACACACTCCCACCCTCCACCCACACT
LeuXAAAxpTrpValSerGlyLysIleSerPheTyrThrLeuProProSerThrHisThr

                130       140       150       160       170       180
                |---------|---------|---------|---------|---------|---
CTGCCTACCCATCTCAGCNCTGAGATTGTTAAGGAGATGACTGAGGTCTTCGACATTGAG
LeuProThrHisLueSerXAAGluIleValLysGluMetThrGluValPheAspIleGlu

                190       200       210       220       230       240
                |---------|---------|---------|---------|---------|---
GATACCGAGCAGGCCAATGAGGACACCATGGAATGCTTGGCCACTGGAGAATCTGATGAG
AspThrGluGlnAlaAsnGluAspThrMetGluCysLeuAlaThrGlyGluSerAspGlu

                250       260       270       280       290       300
                |---------|---------|---------|---------|---------|---
CTGTTGGGTGACATGGACCCGCTTGAAATGGAGATCAAATGGCTCCATTCTCCAATGGTG
LeuLeuGlyAspMetAspProLeuGluMetGluIleLysTrpLeuHisSerProMetVal
```

FIG. 4B


310 320 330 340 350 360
AAATAGCAGATGCCATGGAAAATAAACCACCGTGTATAAGATTGGAGATCTCACTGGG
LysIleAlaAspAlaMetGluAsnLysThrThrValTyrLysIleGlyAspLeuThrGly
370 380 390 400 410 420
TATTGTACCAATCCAAACCGTCATCAGATGGGGTGGGCTAAGCGCAATGTGGACCGACAC
TyrCysThrAsnProAsnArgHisGlnMetGlyTrpAlaLysArgAsnValAspArgHis
430 440
CCCAGAAACAACAGCACACCGGAATTC
ProArgAsnAsnSerThrProGluPhe

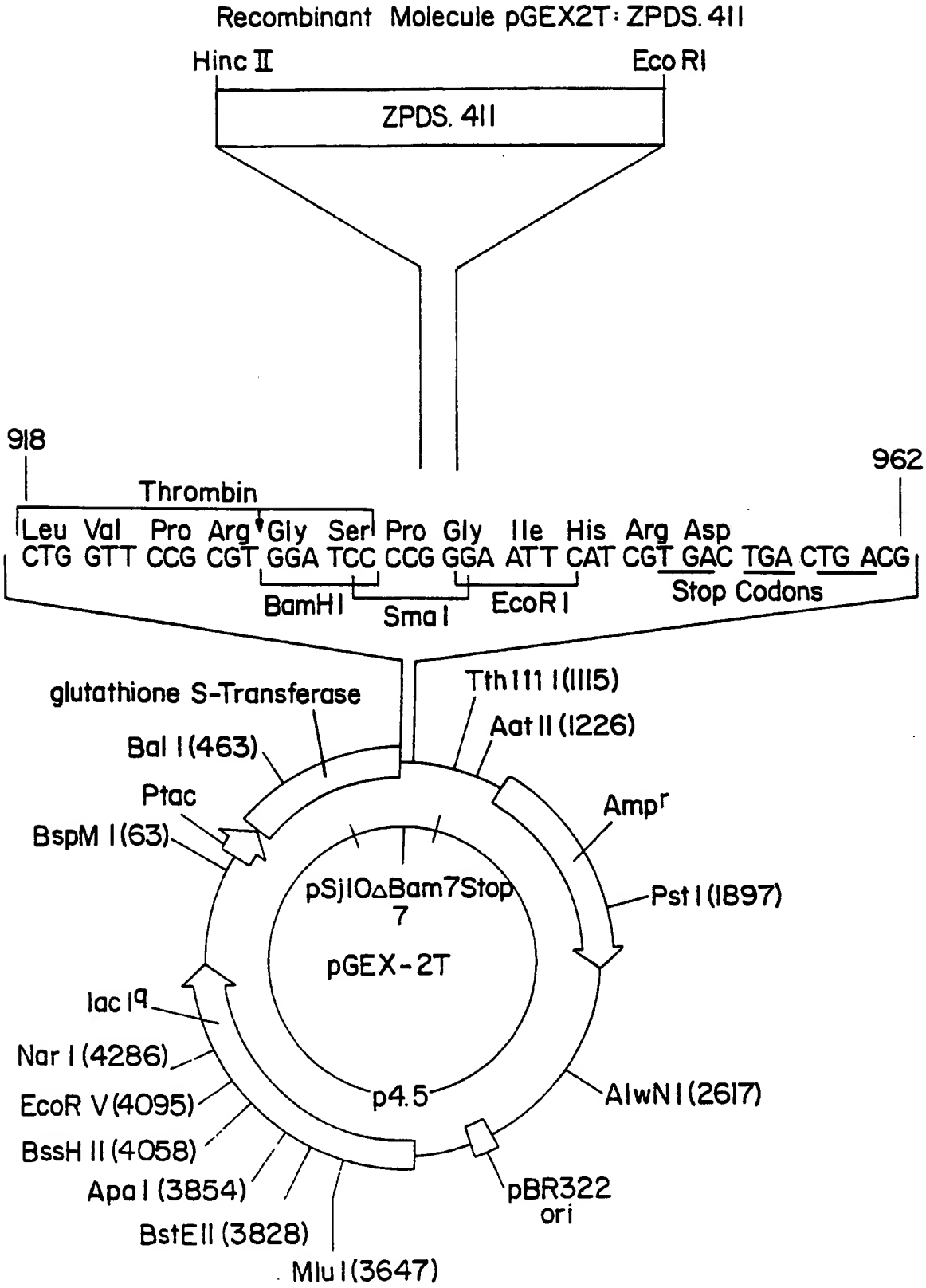

Recombinant Molecule pGEX2T: ZPDS. 411
Hinc II
Eco RI
ZPDS. 411
918
962
Thrombin
Leu Val Pro Arg Gly Ser Pro Gly Ile His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG
BamHI
SmaI
EcoRI
Stop Codons
glutathione S-Transferase
Tth111 I(1115)
Aat II (1226)
Bal I (463)
Ptac
BspM I (63)
Ampr
pSj10ΔBam7Stop
7
pGEX-2T
Pst I (1897)
lac Iq
Nar I (4286)
EcoR V (4095)
BssH II (4058)
Apa I (3854)
BstE II (3828)
Mlu I (3647)
p4.5
AlwN I (2617)
pBR322
ori

FIG. 5

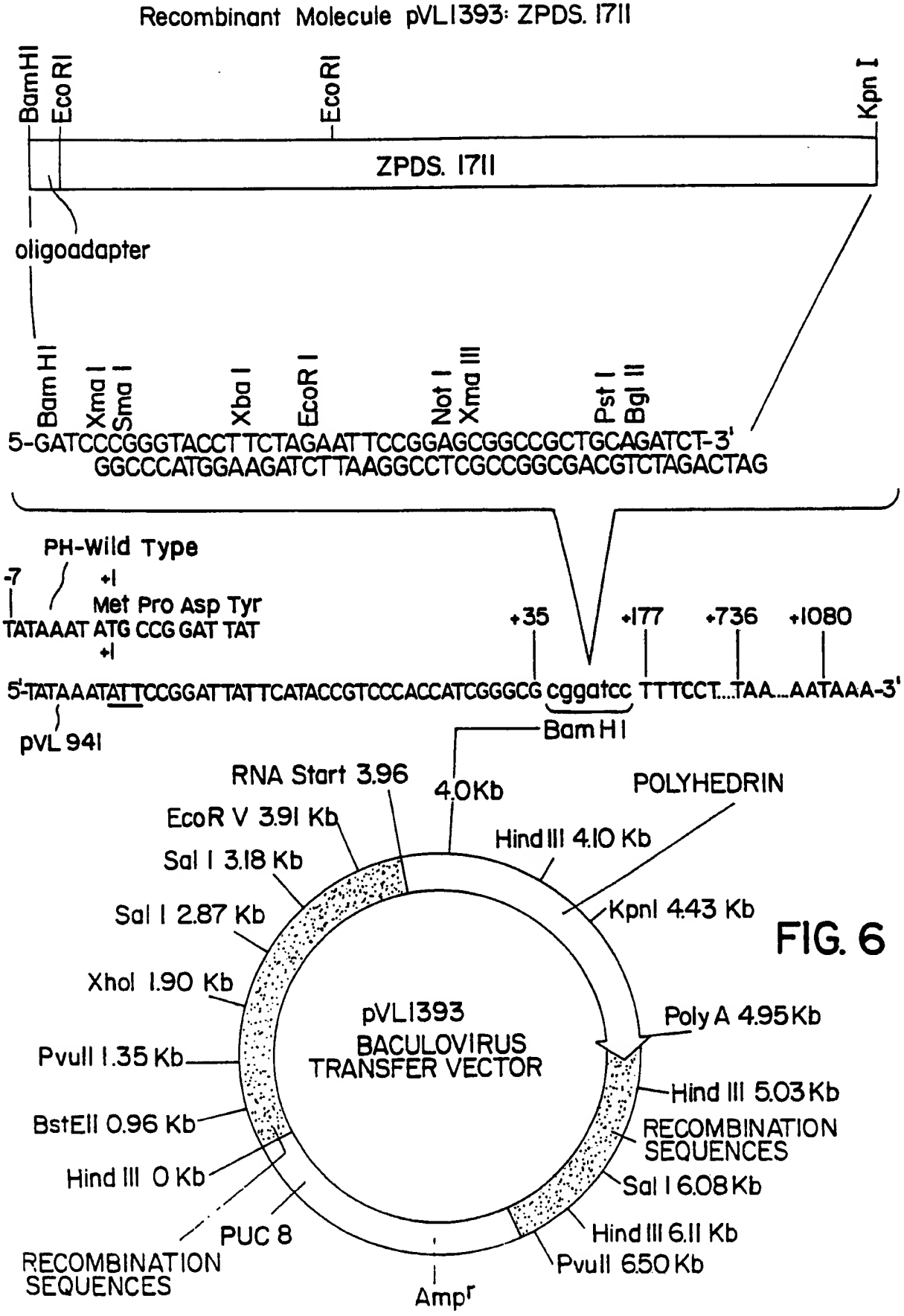

Recombinant Molecule pVL1393: ZPDS. 1711
BamHI
EcoRI
EcoRI
KpnI
ZPDS. 1711
oligoadapter
BamHI
XmaI
SmaI
XbaI
EcoRI
NotI
XmaIII
PstI
BglII
5-GATCCCGGGTACCTTCTAGAATTCCGGAGCGGCCGCTGCAGATCT-3'
GGCCCATGGAAGATCTTAAGGCCTCGCCGGCGACGTCTAGACTAG
PH-Wild Type
-7
+1
Met Pro Asp Tyr
TATAAAT ATG CCG GAT TAT
+1
+35
+177
+736
+1080
5'-TATAAATATTCCGGATTATTCATACCGTCCCACCATCGGGCG cggatcc TTTCCT...TAA...AATAAA-3'
pVL 941
BamHI
RNA Start 3.96
4.0Kb
POLYHEDRIN
EcoR V 3.91 Kb
HindIII 4.10 Kb
Sal I 3.18 Kb
Sal I 2.87 Kb
KpnI 4.43 Kb
XhoI 1.90 Kb
pVL1393
BACULOVIRUS
TRANSFER VECTOR
Poly A 4.95Kb
PvuII 1.35 Kb
HindIII 5.03Kb
BstEII 0.96 Kb
RECOMBINATION
SEQUENCES
HindIII 0 Kb
Sal I 6.08Kb
PUC 8
HindIII 6.11 Kb
RECOMBINATION
SEQUENCES
PvuII 6.50 Kb
Ampr

FIG. 6

INTERNATIONAL SEARCH REPORT

I. national application No.
PCT/US93/01038

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.9, 88, 559; 435/69.3; 530/395, 387.1, 853; 935/9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, CA, EMBASE, APS,
search terms: porcine, zona, pellucida, zp1, zp2, zp3, alpha, beta, glycosylated

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. FAHEY, "MANUAL OF CLINICAL LABORATORY IMMUNOLOGY" published 1986 by AMERICAN SOCIETY FOR MICROBIOLOGY (D.C.), especially pages 99-109.	23, 24
Y	FERTILITY AND STERILITY, Volume 52, Number 2issued AUGUST 1989, B.S. Dunbar et al., "Use of a Synthetic Peptide Adjuvant for the Immunization of Baboons with Denatured and Deglycosylated Pig Zona Pellucida Glycoproteins", pages 311-318, see entire document.	1-60

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 April 1993	Date of mailing of the international search report 09 APR 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer ANTHONY C. CAPUTA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 78, issued 1981, T.P. Hopp et al., "Prediction of Protein Antigenic Determinants from Amino Acid Sequences", pages 3824-3828, see entire document.	1-60
Y	AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, Volume 8, issued 1989, A.G. Sacco et al., "Effect of Varying Dosages and Adjuvants on Antibody Response in Squirrel Monkeys (Saimiri sciureus) Immunized With the Porcine Zona Pellucida, Mr=55,000 Glycoprotein (ZP3)", pages 1-8, see entire document.	1-60
Y	J. SAMBROOK et al., "MOLECULAR CLONING, A LABORATORY MANUAL", published 1989 by COLD SPRING LABORATORY PRESS (N.Y.), pages 16-17.8, especially pages 16.1-16.4, and 17.1-17.8.	1-60
Y	HOPPE-SEYLER'S ZEITSCHRIFT PHYSIOLOGICAL CHEMISTRY, Volume 386, issued APRIL 1982, J. Dietl et al., "Solubilization of four Porcine Zona pellucida Antigens by Lithium 3,5-Diiodosalicylate", pages 381-386, see entire document.	1-60
Y	US, A, 4,745,051 (SMITH ET AL) 17 MAY 1988, see entire document.	1-60
Y	JOURNAL OF REPRODUCTION AND FERTILITY, Volume 76, issued 1986, A.G. Sacco et al., "Carbohydrate influences the immunogenic and antigenic characteristics of the ZP3 macromolecule (Mr 55 000) of the pig zona pellucida", pages 575-585, see entire document.	1-60
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 80, issued MARCH 1983, R.A. YOUNG et al., "Efficient isolation of genes using antibody probes", pages 1194-1198, see entire document.	1-60
P,X P,Y	US, A, 4,996,297 (DUNBAR) 26 FEBRUARY 1991, see entire document.	1-3, 6-7, 10-12, 15, 16, 19-22, 27-29 4, 5, 8, 9, 13, 14, 17, 18, 23-26, 30, 32, 33, 49, 53, 54, 59, 60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01038

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 39/395, 37/02, 37/04, 35/54; C12P 21/06; C07K 15/06, 15/14; C12N 15/10, 15/12

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.9, 88, 559; 435/69.3; 530/395, 387.1, 853; 935/9

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.